Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 333 (2005) 31–40

VIROLOGY

www.elsevier.com/locate/yviro

Internalization and trafficking mechanisms of coxsackievirus B3 in HeLa cells

Sun-Ku Chung[a,b], Joo-Young Kim[a], In-Beom Kim[c], Sang-Ick Park[a], Kyung-Hee Paek[b], Jae-Hwan Nam[a,*]

[a]*Department of Biomedical Sciences, National Institute of Health, 5 Nokbun-dong, Eunpyung-gu, Seoul, 122-701 Korea*
[b]*Graduate School of Biotechnology, Korea University, Seoul, 136-701 Korea*
[c]*Department of Anatomy, College of Medicine, The Catholic University of Korea, Seoul, 137-701 Korea*

Received 30 August 2004; returned to author for revision 22 October 2004; accepted 2 December 2004
Available online 19 January 2005

Abstract

Coxsackievirus B3 (CVB3) is nonenveloped and has a single-stranded positive-sense RNA genome. CVB3 induces myocarditis and ultimately dilated cardiomyopathy. Although there are mounting evidences of an interaction between CVB3 particles and the cellular receptors, coxsackievirus and adenovirus receptor (CAR) and decay-accelerating factor (DAF), very little is known about the mechanisms of internalization and trafficking. In the present study, we used the CVB3 H3 strain, which is CAR-dependent but DAF-independent Woodruff variant and found that during entry, CVB3 particles were colocalized in clathrin, after interacting primarily with CAR, which was not recycled to the plasma membrane. We also found that CVB3 internalization was dependent on the function of dynamin, a large GTPase that has an essential role in endocytosis. Heat-shock cognate protein, Hsc70, which acts as a chaperone in the release of coat proteins from clathrin-coated vesicles (CCV), played a role in CVB3 trafficking processes. Moreover, endosomal acidification was crucial for CVB3 endocytosis. Finally, CVB3 was colocalized in early endosome autoantigen 1 (EEA1) molecules, which are involved in endosome–endosome tethering and fusion. In conclusion, these data together indicate that CVB3 uses clathrin-mediated endocytosis and is transcytosed to early endosomes.

Keywords: CVB3; CAR; Clathrin; Dynamin; Hsc70; Endosomal acidification; EEA1

Introduction

Initial entry of the viral genome and accessory proteins across the plasma membrane and into the cytoplasm of host cells is required to achieve the final goal of viral life to produce progeny by replication and the expression of viral genes. Most viruses use one of two principal methods of internalization. In the first, viruses deliver their genomes into the cytoplasm by direct penetration across the plasma membrane. In the second, the virus itself is invaginated into the cytoplasm by endocytosis using cellular compartments, such as clathrin-coated vesicles (CCV) or caveolae (Pelkmans and Helenius, 2003; Sieczkarski and Whittaker, 2002). Many animal viruses utilize receptor-mediated endocytosis because it has several advantages over the alternative strategy, such as an increase in the efficiency of viral binding to cellular surfaces and a decreased risk of immunodetection (Marsh and Helenius, 1989; Pelkmans and Helenius, 2003).

The entry routes of some viruses have been characterized by observation under electron microscopy (EM), treatment with pharmacological inhibitors of specific entry routes, and the use of dominant-negative mutant proteins involved in specific entry mechanisms (Feig, 1999; Sieczkarski and Whittaker, 2002). Using these methods, it has been established that influenza virus, adenovirus, human poly-

* Corresponding author. Fax: +82 2 388 0924.
E-mail address: jnam66@yahoo.com (J.-H. Nam).

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.12.010

omavirus JC, vesicular stomatitis virus, Semliki Forest virus, Sindbis virus, and human rhinovirus 14 require active clathrin-mediated endocytosis for infection, whereas simian virus 40 and mouse polyomavirus use caveolae-mediated endocytosis (Anderson et al., 1996; DeTulleo and Kirchhausen, 1998; Marsh and Helenius, 1980, 1989; Pho et al., 2000; Superti et al., 1987). More recently, it has been reported that the entry of human papillomavirus (HPV)-16 and HPV-58 involves clathrin-mediated endocytosis, whereas HPV-31 uses caveolae-mediated endocytosis for infection (Bousarghin et al., 2003). It suggests that even strains of the same viral species use different endocytotic mechanisms.

After viruses are entered into the cytoplasm, the internalized viruses use general cell trafficking systems in postendocytic pathways. Therefore, it is reasonable to expect that some cellular factors that play a role in intracellular trafficking may affect viral trafficking. Dynamin, a large GTPase of ~100 kDa, controls the scission reaction that facilitates the budding of invaginated pits from the plasma membrane, which subsequently leads to the formation of clathrin-coated vesicles (CCV), as well as caveolae (Damke et al., 1994; Oh et al., 1998; Sever et al., 2000). Therefore, mutant dynamin confers GTPase deficiency, which specifically blocks endocytosis (Feig, 1999). After the release of coated vesicles, the heat-shock cognate protein, Hsc70, is necessary for the disassembly of the coat protein from the CCV (CCV uncoating process) (Chang et al., 2002; Newmyer and Schmid, 2001; Newmyer et al., 2003). Another important factor for endocytosis is the low-pH-dependent conformational changes that occur within endosomal compartments, which are required for the internalization, uncoating, and trafficking of many viruses. These viruses require a low pH in the intracellular environment for their release from the endocytic pathway into the host cytoplasm (Marsh and Helenius, 1989; Nicola et al., 2003; Sieczkarski and Whittaker, 2002).

Coxsackievirus B3 (CVB3) is a member of the enterovirus genus of the family Picornaviridae. CVB3 is nonenveloped and has a single-stranded positive-sense RNA genome. This virus induces viral myocarditis and ultimately dilated cardiomyopathy (Knowlton and Badorff, 1999). The cellular receptors of CVB3 are known as the coxsackievirus and adenovirus receptor (CAR) and the decay-accelerating factor (DAF; CD55). CAR, in particular, is a major entry receptor for the primary internalization of CVB3, and DAF is a regulatory and attachment protein that acts as a coreceptor (Carson, 2001; Tomko et al., 1997). Previous studies have reported the initial interactions between CVB3 particles and cell-surface receptors (He et al., 2001; Shieh and Bergelson, 2002). However, very little is known of the internalization mechanism or the postendocytotic pathway during or after entry of CVB3.

In the present study, we examined the internalization and trafficking pathway of CVB3 (H3, Woodruff variant) during and after infection. We used several methods, to obviate any ambiguity arising from the method of analysis and to confirm the data of each individual method, which included visual techniques such as confocal microscopy and EM, and the use of mutant proteins or chemical inhibitors relevant to endocytosis. We found that CVB3 interacted initially with CAR, which was not recycled to the plasma membrane. CVB3 also colocalizes with clathrin during entry. Moreover, the use of mutant dynamin and the down-regulation of Hsc70 prevented CVB3 endocytosis in infected HeLa cells. In addition, CVB3 transcytosed to early endosome. The findings described above strongly imply that CVB3 uses CAR in receptor-mediated internalization and clathrin-dependent endocytosis, and that intact endosomal function is necessary for the intracellular trafficking pathway of CVB3.

Results

CVB3 uses CAR as receptor for entry into HeLa cells

Here we used the CVB3 H3 strain of the Woodruff variant for viral infection (referred to herein as CVB3). Although CAR and DAF are known to function as CVB3 receptors, the role of these receptors in CVB3 entry has not been clear. To determine whether anti-CAR or DAF antibodies block CVB3 infection, these antibodies of various dilutions were incubated with cells before CVB3 infection. The blocking of CVB3 infection was clearly dependent on the concentration of anti-CAR, but not anti-DAF (Fig. 1A). Interestingly, anti-DAF-incubated cells also showed partially blocked CVB3 infection (Fig. 1A). Furthermore, only CHO cells expressing CAR could be infected with CVB3, which represented VP1 expression, but not in DAF-expressed CHO cells (Fig. 1B). It means that CAR is major route for CVB3 entry. These results are consistent with previous data (Hsu et al., 1988; Bergelson et al., 1997; Schmidtke et al., 2000). After 30 min or 3.5 h of CVB3 infection, anti-CAR or anti-DAF antibodies were incubated with the cells and detected by flow cytometry (FACS) analysis to calculate the amount of CAR or DAF remaining on the cell surface. FACS analysis showed that CAR was dramatically diminished at 30 min postinfection (pi) and had not recovered by 3.5 h pi, whereas the distribution of DAF did not change at either time point (Fig. 1C). This suggests that CVB3 interacts predominantly with CAR at the cell surface during entry, after which CAR is internalized, but not DAF, with the CVB3 particle. CAR is degraded after internalization and is not recycled to the plasma membrane.

Invaginated CVB3 particles are localized to clathrin-coated vesicles

If CVB3 uses an endocytotic mechanism to infect HeLa cells, it is possible that CVB3 particles are internalized in

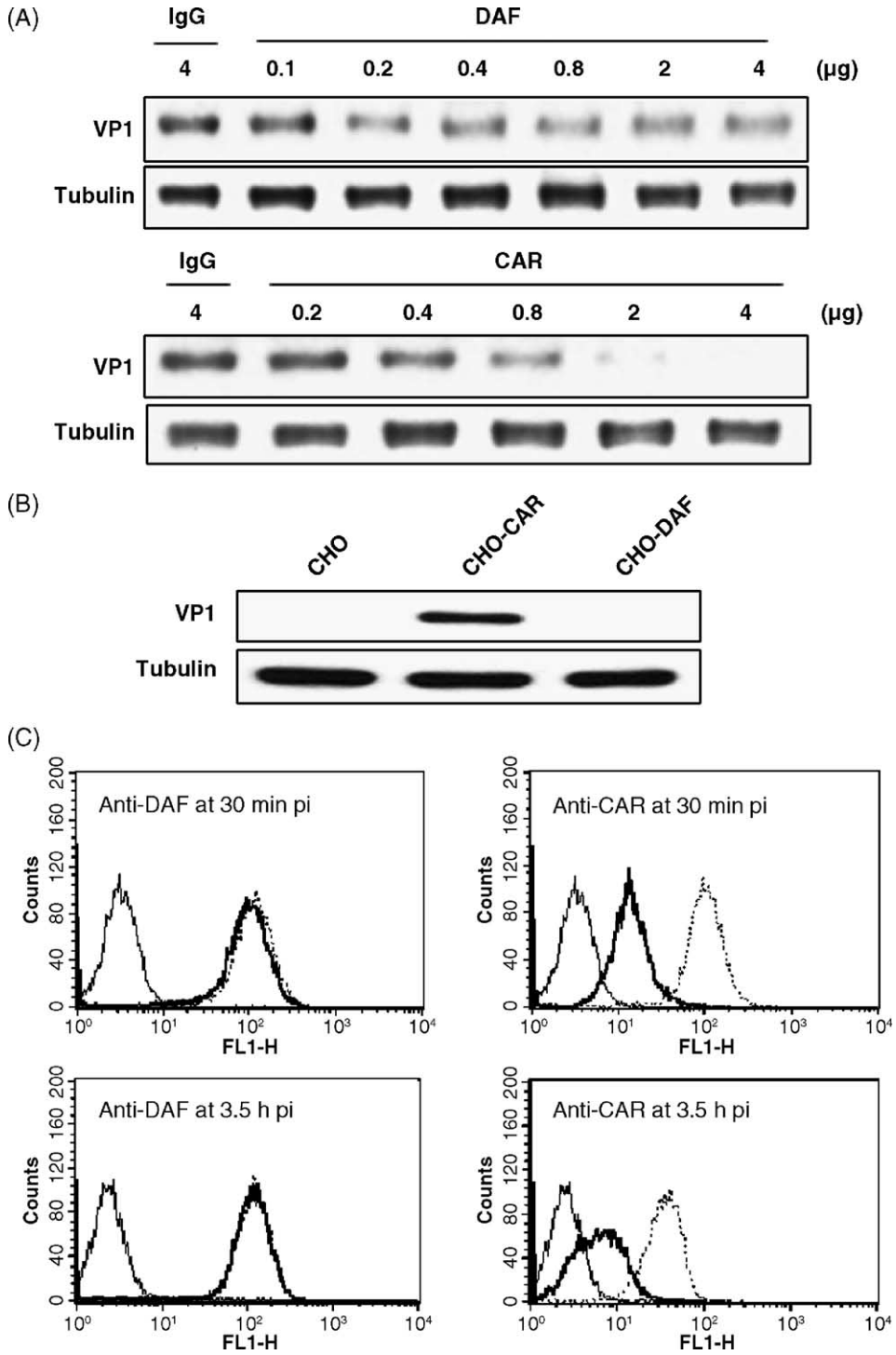

Fig. 1. CVB3 uses CAR as receptor for entry. (A) The effects of blocking CAR or DAF on CVB3 infection. The serial dilution of antibodies to CAR (RmcB, 0.2–4 μg/ml) or DAF (mAb DAF, 0.1–4 μg/ml) were incubated with HeLa cells for 1 h before CVB3 infection. IgG was used for the negative control. At 4 h postinfection (pi), cells were harvested to detect the expression of the CVB3 protein, VP1, with anti-VP1 antibody (dilution 1:1000). Tubulin was used as an internal control. (B) CHO cells were transiently expressed CAR or DAF after 24 h transfection of recombinant plasmids (pcDNA3.1) containing CAR or DAF genes following CMV promoter and infected with CVB3. At 6 h pi, cells were harvested to detect the expression of VP1. (C) Flow-cytometric analysis of CAR and DAF on HeLa cells infected with CVB3 or control HeLa cells without infection at 30 min and 3.5 h pi. The dotted line represents the amount of CAR or DAF on the surfaces of control HeLa cells. The thick line indicates the amount of CAR or DAF on the surfaces of CVB3-infected HeLa cells. The thin line shows the autofluorescent control treated with IgG.

CCV or caveolae via CVB3 receptors during entry. We first examined this possibility using confocal microscopy with an FITC-conjugated monoclonal antibody directed against the viral protein VP1, which is the major external structural protein (visualized as green) and Alexa-Fluor-594-conjugated antibodies directed against clathrin or caveolin, which is abundantly present in caveolae (visualized as red). To increase the possibility of detecting the viral protein VP1 by fluorescence, we infected at a high CVB3 moi (>100) (Fig. 2). At 10 and 20 min pi, viral protein VP1 colocalized with clathrin (Fig. 2A, represented by arrowheads), but not with caveolin (Fig. 2B). Caveolin did not colocalize with VP1 even at 60 min pi (data not shown). Moreover, the distribution of caveolin was clearly different from that of VP1 (Fig. 2B, arrowheads represent caveolin and arrows represent VP1).

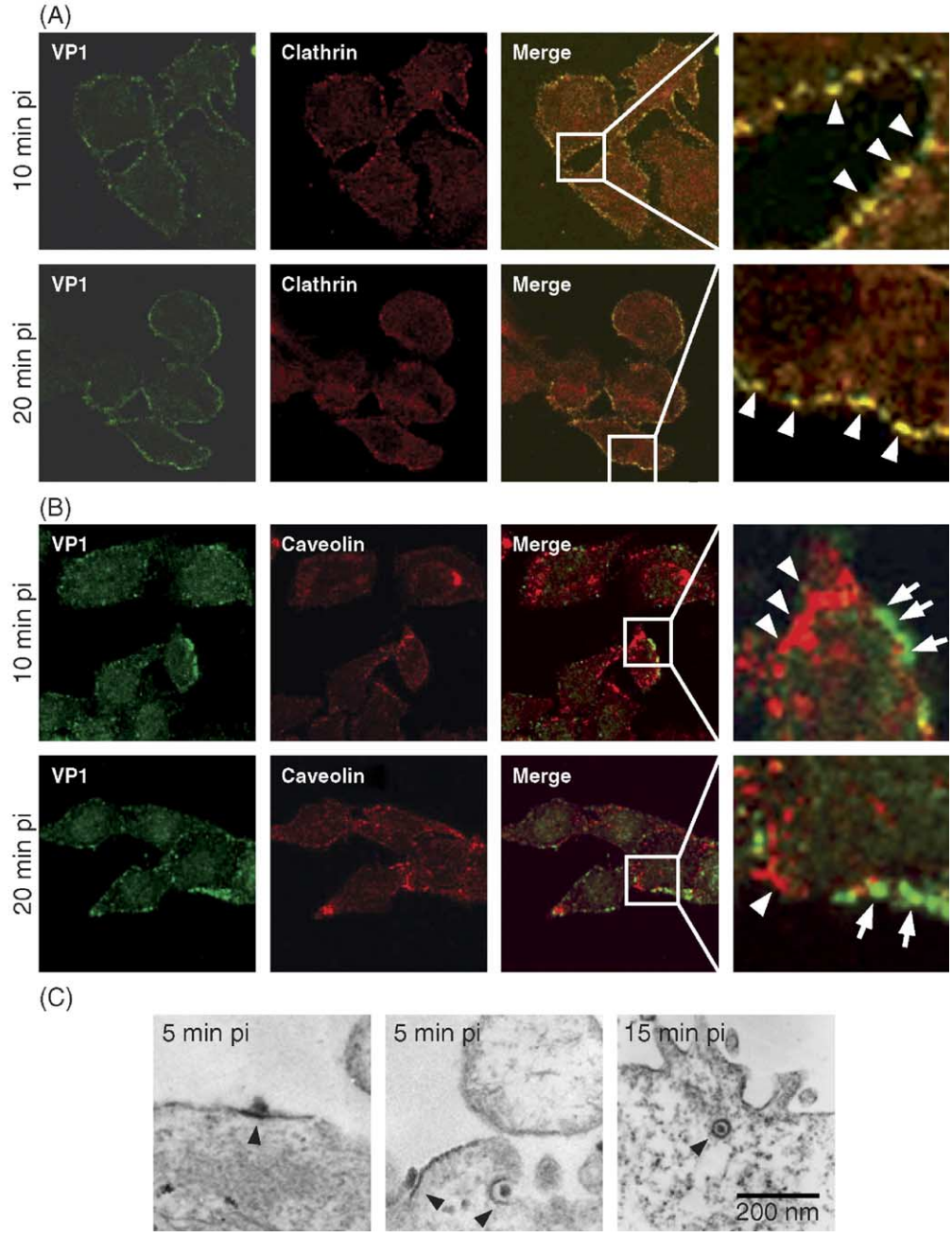

Fig. 2. Internalized CVB3 particles occur in clathrin-coated vesicles. (A) The colocalization of VP1 and clathrin is represented by yellow spots in the merged image, which are indicated by arrowheads. CVB3 protein VP1 is stained with anti-VP1 antibody and FITC-labeled secondary antibody (small green spots). Clathrin is stained with anti-clathrin heavy-chain antibody and Alexa-Fluor-594-labeled secondary antibody (red spots). (B) VP1 (FITC labeled green, indicated by arrows) and caveolin-1 (Alexa-Fluor-594 labeled red, indicated by arrowheads) present in caveolae do not colocalize in the merged image. (C) Electron microscopic analysis of CVB3 uptake into HeLa cells. The section is immunostained with anti-clathrin antibody. Arrowheads on the left and middle indicate a forming clathrin-coated pit (CCP) with CVB3. Arrowhead on the right represents invaginated CVB3 particles in a clathrin-coated vesicle (CCV).

To confirm the confocal microscopy data that demonstrated the colocalization of CVB3 particles and clathrin, we used immunoelectron microscopy staining for clathrin. CVB3-infected cells were incubated for 5 and 15 min pi before fixation. At 5 min pi, some CVB3 particles were bound to newly forming clathrin-coated pits (CCP) (Fig. 2C left and middle, represented by arrowheads). At 15 min pi, viral particles were present in CCV (Fig. 2C right, represented by arrowhead). Although there are some limitations to the interpretation of EM data, such as sectioning artifacts and the rapid removal of clathrin coats upon endocytosis (Ashbourne Excoffon et al., 2003), the results of confocal microscopy and EM clearly suggest that the internalization of CVB3 across the plasma membrane occurs via a clathrin-mediated mechanism.

Dynamin is necessary for CVB3 endocytosis

Dynamin, a large GTPase, is apparently required in clathrin- or caveolae-mediated endocytosis (Damke et al., 1994; Oh et al., 1998). This protein regulates the fission reaction that forms vesicles from the plasma membrane and also plays a role in the actin-mediated transport of endocytic vesicles via a GTP-dependent mechanism (Jin et al., 2002; Lee and De Camilli, 2002; Sever et al., 2000). A dynamin mutant, K44A, is defective in GTP binding and blocks endocytosis (DeTulleo and Kirchhausen, 1998; Jin et al., 2002). In this study, we used two types of HeLa cells that had been stably transfected with either wild-type or dominant-negative (K44A) dynamin controlled by a tetracycline-inducible promoter. Both types of HeLa cells expressed dynamin during incubation in tetracycline-deficient medium. After 2 days incubation in tetracycline-deficient medium, cells were infected with CVB3. At 4 h pi, infected cells were harvested and analyzed by Western blots probed with anti-VP1 antibody to measure the infectivity of CVB3 in both cell types. We confirmed that the expression of VP1 is well represented the virus titer, which is achieved by plaque assay (data not shown). Therefore, we used the expression of VP1 as index of the infectivity of CVB3 in this study. We found that the infectivity of CVB3 in K44A-transfected cells was reduced compared with that of HeLa cells expressing wild-type dynamin (Fig. 3A). K44A did not completely block CVB3 infection because K44A was not expressed in 40–60% of cells, which is supported by a previous report (Jin et al., 2002). About half the K44A-transfected cells expressed wild-type dynamin, so CVB3 could infect these cells. Therefore, a 60–70% reduction in infectivity is sufficient to imply that the infection of CVB3 is dependent on dynamin-regulated endocytosis.

Hsc70 is involved in CVB3 trafficking

There are mounting evidences that Hsc70 regulates coat disassembly and the multiple transport and sorting events

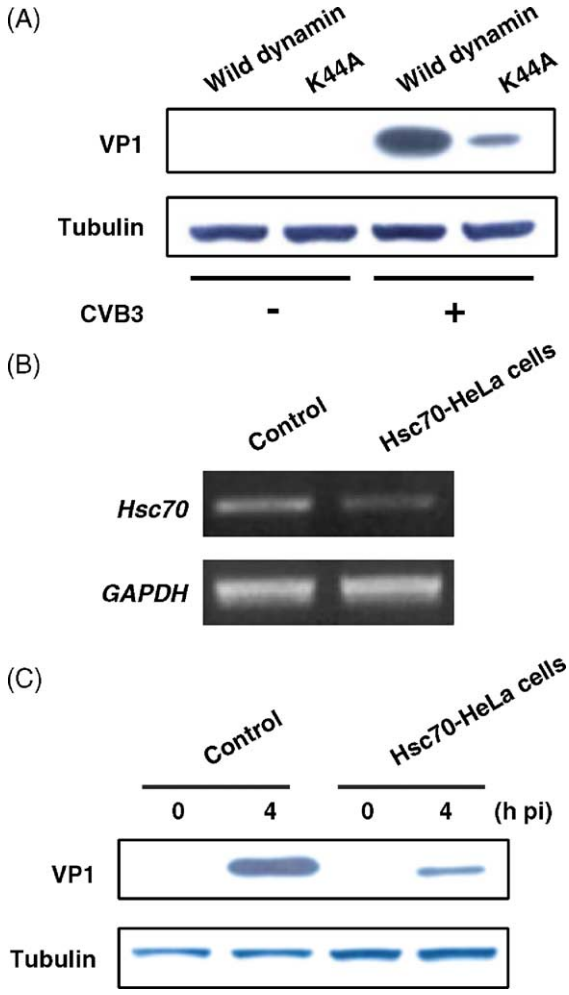

Fig. 3. Dynamin and Hsc70 proteins are critical to CVB3 endocytosis. (A) Effect of dynamin on CVB3 infection of HeLa cells. Four hours after CVB3 infection of sham-infected, wild-type (expressing wild-type dynamin), and K44A (expressing mutant dynamin) HeLa cells, the cells were harvested and the level of VP1 expression determined as an index of the efficiency of CVB3 infection. All cells were cultured in tetracycline-deficient medium to induce wild-type or mutant dynamin. (B) Effects of *hsc70* siRNA on HeLa cells. Plasmid encoding *hsc70* siRNA or siRNA empty vector (for details, see Materials and methods) was transfected into cells, and transfectants were selected with hygromycin to produce stable cell lines. *hsc70* mRNA levels were detected by RT–PCR. HeLa cells transfected with siRNA empty vector were used as controls. *GAPDH* was used as the internal RNA control. (C) Hsc70 effect on CVB3 infection. Hsc70–HeLa cells are HeLa cells with reduced levels of *hsc70* mRNA generated with siRNA. At 4 h pi with CVB3, cells were harvested and the levels of VP1 expression determined as an index of the efficiency of CVB3 infection. Tubulin was used as an internal control.

between endosomal compartments, in that Hsc70 broadly modulates clathrin dynamics throughout the CCV cycle (Chang et al., 2002; Newmyer and Schmid, 2001; Newmyer et al., 2003). Moreover, it was previously reported that antibodies against Hsc70 interfered with receptor-mediated endocytosis (Honing et al., 1994). Therefore, if Hsc70 has a role in CVB3 endocytosis, it means that invaginated CVB3 particles are related with CCV. Therefore, we explored whether Hsc70 is necessary for CVB3 endocytosis. Firstly,

stable HeLa cells with down-regulated *hsc70* gene expression (hsc70–HeLa cells) were generated with small-interfering RNAs (siRNA), and we verified that *hsc70* RNA levels were down-regulated in these cells (Fig. 3B). When infected with CVB3, the infectivity of CVB3 in hsc70–HeLa cells was lower than in control cells (HeLa cells transfected with siRNA empty vector, p*Silencer*™ 2.1), corresponding to the reduction in *hsc70* RNA levels (Fig. 3C). This suggests that Hsc70 is critically involved in CVB3 endocytosis, and therefore that CVB3 endocytosis is dependent upon the CCV cycle.

CVB3 trafficking is significantly dependent on endosomal acidification

Some viral endocytosis is dependent on the low-pH-mediated conformational changes that trigger fusion, penetration, or uncoating (Sieczkarski and Whittaker, 2002). To determine whether CVB3 is dependent upon low pH in trafficking in cytosol, we treated cells with NH_4Cl before viral infection. NH_4Cl is a lysosomotropic agent that causes a rapid rise in the pH of intracellular organelles including endosomes, inhibiting low-pH-dependent cellular processes such as endosomal fusion. Therefore, NH_4Cl has been used broadly to study the endosomal acidification for endocytosis/trafficking (Nicola et al., 2003; Ohkuma and Poole, 1978). In the NH_4Cl inhibition test, cells were pretreated with the concentration of NH_4Cl (50 mM) for 30 min prior to infection with CVB3 at a moi of 10. At 4 h pi, the expression of viral proteins for progeny assembly was clearly apparent in mock cells (Fig. 4A, represented by FITC-stained green regions). However, there was no expression of new viral proteins in NH_4Cl-treated cells, whereas a few viral particles were trapped in CCV during NH_4Cl treatment (Fig. 4B, represented by yellow spots indicated with arrowheads; VP1 represented by green spots, clathrin represented by red spots). This suggests that the fusion of CCV and early endosomes is dependent on low pH. Based on the above data (Fig. 4), CVB3 trafficking in cytosol after entry is significantly dependent on low-pH environments in cellular organelles.

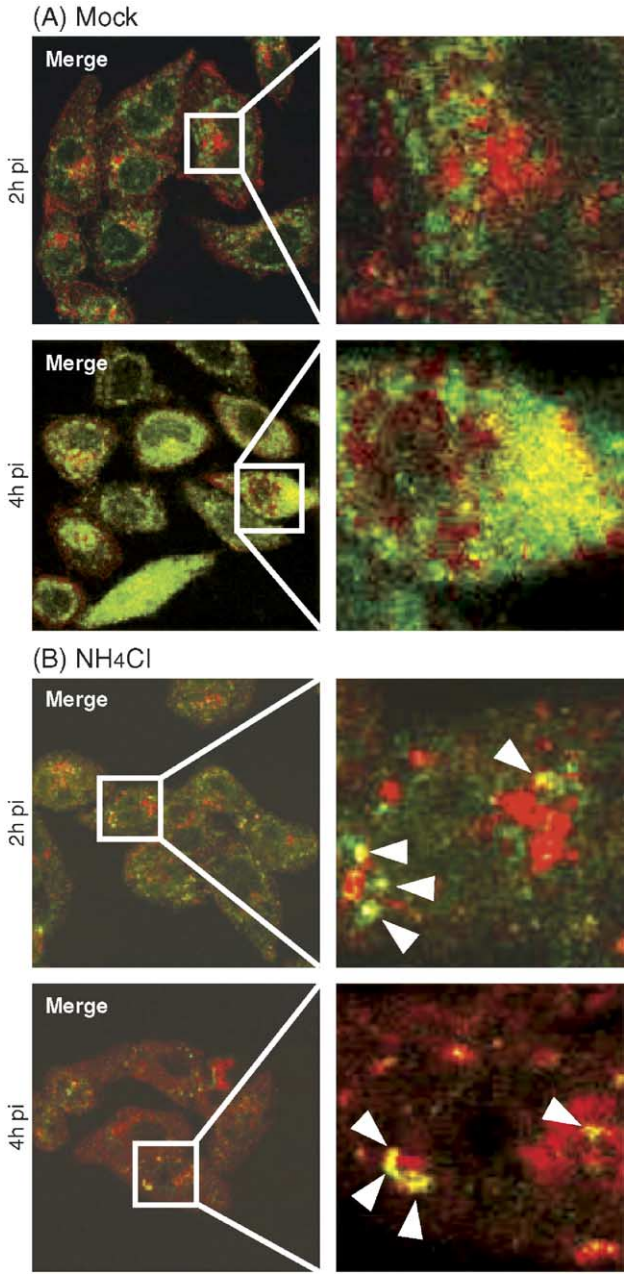

Fig. 4. Low pH in cellular organelles is critical for CVB3 infection. HeLa cells were treated with 50 mM of NH_4Cl for 30 min before CVB3 infection (moi = 10). (A) Distribution and expression of CVB3 VP1 during infection of mock-treated cells (not treated with NH_4Cl) at 2 and 4 h pi. De novo synthesis of VP1 protein in progeny viruses at 4 h pi is shown as green regions (FITC label). Alexa Fluor 594 (red) indicates clathrin. (B) Effects of NH_4Cl on the distribution and expression of CVB3 VP1. HeLa cells were treated with NH_4Cl (50 mM) before CVB3 infection, and NH_4Cl levels were maintained during and after infection. No synthesis of VP1 or progeny viruses was observed even at 4 h pi. Compare with panel A images. Internalized viruses were trapped in CCV, which are represented by yellow spots (indicated by arrowheads). Green spots represent FITC-labeled VP1 and red spots represent Alexa-Fluor-568-labeled clathrin.

Internalized CVB3 is transcytosed to endosomes

The results described above (Fig. 4), showing that the maintenance of a low pH in cellular organelles, such as endosomes, is critical, indicates that endosomes are physically involved in CVB3 endocytosis. To confirm this, we stained CVB3 particles with anti-VP1 antibody and endosomes with antibody directed against EEA1, a cytosolic protein that may be associated with early endosomal membranes and that is involved in endosome–endosome tethering and fusion (Simonsen et al., 1998), in infected HeLa cells and followed the signals over time. CVB3 particles clearly colocalized with early endosomes (Fig. 5, represented by yellow spots indicated with arrowheads). We identified some colocalizing yellow spots at 1.5 h pi and 3 h pi (Fig. 5), indicating that the

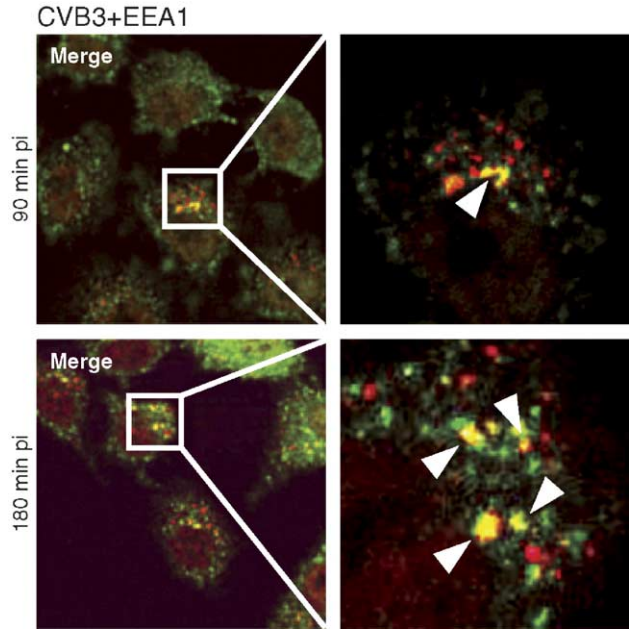

Fig. 5. Internalized CVB3 is transcytosed to early endosomes. Colocalization of VP1 and EEA1 is indicated by arrowheads. CVB3 VP1 is represented by FITC label (green spots). Alexa Fluor 594 label (red spots) represents EEA1.

CCV containing internalized CVB3 particles fused with early endosomes.

Discussion

It is generally accepted that once delivered to early endosomes, recycling receptors are returned to the cell surface, in contrast to down-regulated receptors, which are transported to late endosomes and thence to lysosomes (Gruenberg and Maxfield, 1995). Our initial experiments described here (Fig. 1) show the role of CAR in CVB3 endocytosis. CVB3 interacts with CAR to cross the plasma membrane. CAR is internalized with CVB3 particles and does not return to the cell surface via recycling endosomes as some other cellular receptors do, such as the GPI-anchored protein, DAF (Gruenberg and Maxfield, 1995; Sabharanjak et al., 2002). In these experiments, we could exclude the possibility in which CVB3 masks CAR molecules on the cell surface to block the binding of anti-CAR to CAR because CVB3 clearly crosses the plasma membrane and is present inside cells less than 10 min pi (as shown in Fig. 2) and the trypsin treatment before immunostaining for FACS analysis dissociated the binding remained CVB3 particles and cellular receptor on cell surface. Therefore, the above data (Fig. 1) strongly suggest that CAR acts as a major receptor interacting with CVB3 on the cell surface and that this interacting complex is invaginated in CCV. The internalized CAR is not recycled to the cell surface.

Here we used Western blotting to detect viral protein expression in infected cells. To confirm whether the Western blot data could represent a productive infection, we infected with UV-inactivated virus and activated virus (100 moi) for 1 h and washed with PBS. Then we performed Western blotting with anti-VP1 at 4 h and 6 h pi. UV-inactivated virus infection did not show any VP1 band (data not shown). Therefore, it is possible that Western blot detection represents viral infection and expression.

Visual images provided by confocal microscopy and EM analysis show that the internalized CVB3 particles are clearly present in CCV (Fig. 2). To confirm it, we explored whether some endocytosis-related factors play a role in the internalization and trafficking of CVB3, especially clathrin-mediated endocytosis, over a sequential time course. When clathrin-coated pits (CCP) become CCV, dynamin functions to cut the neck of the CCP to form CCV; it also acts in this capacity in the formation of caveolae (Damke et al., 1994; Oh et al., 1998). Here, the infectivity of CVB3 was reduced in K44A cells containing in a dynamin mutant (Fig. 3A), suggesting that the entry of CVB3 requires an intact dynamin function and that the entry of CVB3 is mediated by endocytosis, and not by direct delivery of the viral genome on the cell surface.

The data described above demonstrate that the internalized CVB3 particles are present in CCV. The fusion of these CCV with early endosomes requires clathrin uncoating of the CCV, which is regulated by Hsc70 activity (Chang et al., 2002; Newmyer et al., 2003). Therefore, the Hsc70 requirement for CVB3 infection, based on Fig. 3C, supports the proposition that CVB3 is internalized by clathrin-mediated endocytosis. Moreover, as we show in Fig. 4, in CVB3 infection, the acidic environments of cellular organelles are critically necessary for the fusion of CCV to early endosomes and for the fusion of endosomes to endosomes, as has been described for adenovirus and herpes simplex virus (Da Costa et al., 2003; Meier and Greber, 2003; Nicola et al., 2003). Finally, we clearly confirmed that internalized CVB3 colocalizes with EEA1, which is associated with early endosomes (Fig. 5). These results strongly suggest that the internalized CVB3 particles are transcytosed to early endosomes.

Very recently, Triantafilou and Triantafilou (2004) reported that CVB4, which is implicated in the development of insulin-dependent diabetes mellitus (Horwitz, 1990), is endocytosed independently of clathrin-associated machinery and is delivered to the Golgi via a lipid-raft-dependent mechanism. Actually, strain-specific differences of CVB1-6 including the interaction of CVBs with CAR and DAF, and the cytopathic effects in several cell types, have been well studied (Selinka et al., 2002; Schmidtke et al., 2000; Pasch et al., 1999). Furthermore, the extensive differences of interaction with CAR and DAF could occur even within the same serotype group of CVB3 and these differences may have a role in the different virulent phenotypes of the CVB3 strains (Selinka et al., 2002; Schmidtke et al., 2000). Previously, it has been published that anti-DAF blocked infection with CVB1 (Conn-5), CVB3 (Nancy), and CVB5

(Faulkner) while anti-CAR blocked infection with all six CVB strains (Bergelson et al., 1997; Wang and Bergelson, 1999). However, the entry of CVB3 H3 strain, originating from the Woodruff variant, is dependent on CAR, but not DAF (Fig. 1). Therefore, it is possible that the endocytotic mechanism of CVB3 (H3) and CVB4 (Edwards strain 2, E2) could be distinct. These differences of endocytosis mechanism in the same viral strains are also found in human papilloma viruses (HPVs) where HPV-16 and -58 used clathrin-dependent endocytosis while HPV-31 used caveolae-dependent endocytosis (Bousarghin et al., 2003).

In here, we used the visual images, which were achieved by confocal microscopy and EM, and the mutants or chemical inhibition of several cellular factors, which were related with endocytosis to explore the viral internalization and trafficking, although some is not shown directly. However, because all factors tested in this study were previously reported the relation with endocytosis and all data were complementary for each other, it is clear to determine the endocytosis mechanism of CVB3.

Based on these results taken together, we propose an endocytic model for CVB3 H3 Woodruff variant, in which CVB3 is internalized, after binding to the receptor CAR, and is involved with clathrin. The internalized CVB3 particles in CCV are then transcytosed into early endosomes by a trafficking process.

Materials and methods

Cells and virus

The cervical cancer cell line HeLa–UVM and Chinese hamster ovary (CHO) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The HeLa–UVM cell line (referred to herein as HeLa cells) was obtained from Sally Huber (University of Vermont, USA). CVB3, which is an H3 strain (Woodruff variant; the generous gift of E. Jeon, Samsung Medical Center, Korea; Knowlton et al., 1996), was grown and titered using HeLa cells. Viral stocks were prepared by infecting 90% confluent cultures of HeLa cells at a multiplicity of infection (moi) of 10. After incubation for 16 h at 37 °C, the suspension was freeze-thawed three times. The cell lysates were centrifuged, and the supernatants were harvested and stored at −75 °C. The virus titer was measured by plaque assay. The titer of CVB3 H3 was 1×10^9 plaque-forming units (pfu)/ml. A stock of radioactively labeled CVB3 was prepared as previously described (Zaragoza et al., 1997). The pellet was resuspended in phosphate-buffered saline (PBS) and the viral titer was measured. The labeled viral stock used in this work had a titer of 2×10^8 pfu/ml. HeLa cells stably expressing mutant dynamin (K44A) or wild-type dynamin were kindly supplied by K. Ahn (Division of Life Science and Graduate School of Biotechnology, Korea University, Korea). The cells were cultured in DMEM supplemented with 10% FBS, 400 μg/ml G418 (Life Technologies, Inc.), 200 ng/ml puromycin, and 1 μg/ml tetracycline. Cells were subcultured in six-well plates. For induction, cells were grown in the absence of tetracycline for 48 h at 37 °C before use. The cells were grown to 80% confluence.

Antibodies and reagents

Anti-CAR antibody (clone RmcB) directed against hCAR was purchased from Upstate Biotech. Mouse monoclonal antibody directed against human DAF (CD55; mAb DAF) was from BD Pharmingen. Mouse mAb ncl-entero (anti-VP1) against an epitope on the VP1 peptide was obtained from Novocastra Laboratories. Anti-CHC (anti-clathrin) monoclonal antibody was obtained from BD Pharmingen. Rabbit polyclonal antibody directed against caveolin-1 was also obtained from BD Pharmingen. Rabbit polyclonal antibody directed against EEA1 was obtained from Abcam. Anti-mouse IgG-fluorescein isothiocyanate (FITC) and anti-rabbit IgG-FITC were purchased from Santa Cruz Biotechnology. Alexa-Fluor-594-conjugated mouse IgG and rabbit IgG were obtained from Molecular Probes Inc. Alexa-Fluor-594-phalloidin (anti-actin) was obtained from Molecular Probes Inc.

Flow-cytometric analysis

After viral infection, cells were harvested in trypsin-EDTA, pooled, and resuspended in fresh medium (DMEM) supplemented with 10% FBS. After brief centrifugation ($340 \times g$) for 5 min at 4 °C, the supernatant was discarded and the cells were washed in PBS containing 1% BSA (PBA). The cells were resuspended with primary antibody (1:100) and incubated on ice for 60 min. Cell pellets were then washed in PBA and incubated with mouse IgG-FITC (1:200) for 40 min. The cells were washed twice more with PBS. The expression of CAR and DAF in suspended cell samples was directly determined using a FACSCalibur cytometer (Becton Dickinson). Ten thousand fluorescent events per sample were acquired using a 530/15 band-pass filter for the FITC signal with fluorescence emission centered at 530 nm.

Immunofluorescence

In the presence or absence of inhibitor, 60% confluent monolayers of HeLa cells were grown on glass coverslips. The cells were then infected at a moi of >100 CVB3 at 37 °C for various times. The cells on glass coverslips were fixed with 4% paraformaldehyde for 15 min, quenched with 50 mM NH_4Cl for 10 min, and then permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were washed, blocked with PBA (containing 2% BSA), and incubated with primary antibody (1:50–1:100 dilution) for 40 min. After this time, the coverslips were

washed three times with PBA and then incubated for 30 min with Alexa Fluor 488 or Alexa Fluor 594 (1:100; Molecular Probes Inc.). The coverslips were then washed three times and mounted onto glass slides with fluorescent mounting medium (Dako Corp.). Images of cells were viewed using a Bio-Rad LaserSharp confocal microscope.

Electron microscopy

Confluent cells were cultured in six-well plates (Nunc). Infection with CVB3 was performed at a moi of 100. At different time points (5 and 15 min) after infection, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4), for 20 min at 4 °C. Following fixation, the cells were washed three times with 0.01 M phosphate-buffered saline (PBS; pH 7.4). They were incubated in 10% normal goat serum (NGS) in PBS for 1 h at room temperature, to block nonspecific binding, and were then incubated in anti-clathrin (anti-CHC) sera for 12 h at 4 °C. The cells were washed in PBS for 45 min (3 × 15 min), incubated in biotin-labeled goat anti-mouse (Jackson Immuno Research; dilution 1:100) for 1 h, and then washed three times in PBS for 45 min (3 × 15 min). The cells were then incubated in avidin–biotin–peroxidase complex (ABC) solution (Vector Laboratories, Burlingame, CA) for 1 h, washed in TB, and then preincubated in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.1 M Tris buffer for 10 min and incubated in the same solution containing 0.05% hydrogen peroxide (H_2O_2) for an additional 10 min. Stained sections were post-fixed in 1% glutaraldehyde in PB for 1 h and, after washing in PB containing 4.5% sucrose for 15 min (3 × 5 min), they were post-fixed in 1% osmium tetroxide (OsO4) in PB for 1 h. Afterwards they were rewashed in PB containing 4.5% sucrose and dehydrated in a graded series of alcohol. During the dehydration procedure, they were stained with 1% uranyl acetate in 70% alcohol for 1 h, then transferred to propylene oxide, and embedded in Epon 812. After curing at 60 °C for 3 days, the Epon blocks were cut out and attached to an Epon support for further ultrathin sectioning (Reichert-Jung, Nuâloch, Germany). Ultrathin sections (70–90 nm thick) were collected on one-hole grids coated with Formvar and examined using an electron microscope (Jeol 1200EX, Tokyo, Japan).

Western blotting

Cells infected by virus in the absence or presence of various chemicals were washed with ice-cold Dulbecco's phosphate-buffered saline and lysed in lysis buffer, as described above, containing 1 × Protease Inhibitor Mixture (Roche Applied Science). Protein concentrations were determined with a BCA Assay Kit (Sigma). Equal quantities of protein (30 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes. The blots were then probed with the appropriate antibody directed against VP1 (1:1000), hCAR (1:500), or EEA1 (1:1000), followed by incubation with the corresponding secondary antibody, and visualization with ECL reagents (Perbio).

Construction of small interfering RNA (siRNA) for hsc70

The target sequence for *hsc70* siRNA is as follows: 5′-GATCCGTCGAGATAATTGCCAATGTTCAAGAGA-CATTGGCAATTATCTCGACTTTTTTGGAAA-3′. The underlined sequence represents the target regions of *hsc70* mRNA. This siRNA was cloned downstream from the human U6 promoter as described in the p*Silencer*™ 2.1-U6 hygro manual (Ambion). For transfection, cells were plated onto dishes at 70–80% confluence without antibiotics. This plasmid construct was transfected into HeLa cells at a concentration of 400 ng siRNA per 35 mm dish with Lipofectamine Reagent (Invitrogen), as directed by the manufacturer.

Acknowledgments

J.-H. Nam and K.-H. Paek contributed equally to this work. We thank Dr. Ellie Ehrenfeld for reviewing the manuscript and Drs. Judith Graff and Rajen Koshy for critical advises for this study. This work was supported by intramural fund of National Institute of Health, Korea (348-6111-213-000-207).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2004.12.010.

References

Anderson, H.A., Chen, Y., Norkin, L.C., 1996. Bound simian virus 40 translocates to caveolin-enriched membrane domains, and its entry is inhibited by drugs that selectively disrupt caveolae. Mol. Biol. Cell 7, 1825–1834.

Ashbourne Excoffon, K.J., Moninger, T., Zabner, J., 2003. The coxsackie B virus and adenovirus receptor resides in a distinct membrane microdomain. J. Virol. 77, 2559–2567.

Bergelson, J.M., Cunningham, J.A., Droguett, G., Kurt-Jones, E.A., Krithivas, A., Hong, J.S., Horwitz, M.S., Crowell, R.L., Finberg, R.W., 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. Science 275, 1320–1323.

Bousarghin, L., Touze, A., Sizaret, P.Y., Coursaget, P., 2003. Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. J. Virol. 77, 3846–3850.

Carson, S.D., 2001. Receptor for the group B coxsackieviruses and adenoviruses: CAR. Rev. Med. Virol. 11, 219–226.

Chang, H.C., Newmyer, S.L., Hull, M.J., Ebersold, M., Schmid, S.L.,

Orsi, N., 2002. Hsc70 is required for endocytosis and clathrin function in Drosophila. J. Cell Biol. 159, 477–487.

Da Costa, S.R., Sou, E., Xie, J., Yarber, F.A., Okamoto, C.T., Pidgeon, M., Kessels, M.M., Mircheff, A.K., Schechter, J.E., Qualmann, B., Hamm-Alvarez, S.F., 2003. Impairing actin filament or syndapin functions promotes accumulation of clathrin-coated vesicles at the apical plasma membrane of acinar epithelial cells. Mol. Biol. Cell 14, 4397–4413.

Damke, H., Baba, T., Warnock, D.E., Schmid, S.L., 1994. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. J. Cell Biol. 127, 915–934.

DeTulleo, L., Kirchhausen, T., 1998. Clathrin endocytic pathway in viral infection. EMBO J. 17, 4585–4593.

Feig, L.A., 1999. Tools of the trade: use of dominant-inhibitory mutants of Ras-family GTPases. Nat. Cell Biol. 1, E25–E27.

Gruenberg, J., Maxfield, F.R., 1995. Membrane transport in the endocytic pathway. Curr. Opin. Cell Biol. 7, 552–563.

He, Y., Chipman, P.R., Howitt, J., Bator, C.M., Whitt, M.A., Baker, T.S., Kuhn, R.J., Anderson, C.W., Freimuth, P., Rossmann, M.G., 2001. Interaction of coxsackievirus B3 with the full length coxsackievirus-adenovirus receptor. Nat. Struct. Biol. 8, 874–878.

Honing, S., Kreimer, G., Robenek, H., Jockusch, B.M., 1994. Receptor-mediated endocytosis is sensitive to antibodies against the uncoating ATPase (hsc70). J. Cell Sci. 107, 1185–1196.

Horwitz, M., 1990. The adenoviridae and their replication. In: Fields, B.N., Knipe, D.M. (Eds.), Virology. Raven Press, New York, pp. 1679–1721.

Hsu, K.H., Lonberg-Holm, K., Alstein, B., Crowell, R.L., 1988. A monoclonal antibody specific for the cellular receptor for the group B coxsackieviruses. J. Virol. 62, 1647–1652.

Jin, M., Park, J., Lee, S., Park, B., Shin, J., Song, K.J., Ahn, T.I., Hwang, S.Y., Ahn, B.Y., Ahn, K., 2002. Hantaan virus enters cells by clathrin-dependent receptor-mediated endocytosis. Virology 294, 60–69.

Knowlton, K.U., Badorff, C., 1999. The immune system in viral myocarditis: maintaining the balance. Circ. Res. 85, 559–561.

Knowlton, K.U., Jeon, E.S., Berkley, N., Wessely, R., Huber, S., 1996. A mutation in the puff region of VP2 attenuates the myocarditic phenotype of an infectious cDNA of the Woodruff variant of coxsackievirus B3. J. Virol. 70, 7811–7818.

Lee, E., De Camilli, P., 2002. Dynamin at actin tails. Proc. Natl. Acad. Sci. U.S.A. 99, 161–166.

Marsh, M., Helenius, A., 1980. Adsorptive endocytosis of Semliki Forest virus. J. Mol. Biol. 142, 439–454.

Marsh, M., Helenius, A., 1989. Virus entry into animal cells. Adv. Virus Res. 36, 107–151.

Meier, O., Greber, U.F., 2003. Adenovirus endocytosis. J. Gene Med. 5, 451–462.

Newmyer, S.L., Schmid, S.L., 2001. Dominant-interfering Hsc70 mutants disrupt multiple stages of the clathrin-coated vesicle cycle in vivo. J. Cell Biol. 152, 607–620.

Newmyer, S.L., Christensen, A., Sever, S., 2003. Auxilin–dynamin interactions link the uncoating ATPase chaperone machinery with vesicle formation. Dev. Cell 4, 929–940.

Nicola, A.V., McEvoy, A.M., Straus, S.E., 2003. Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells. J. Virol. 77, 5324–5332.

Ohkuma, S., Poole, B., 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U.S.A. 75, 3327–3331.

Oh, P., McIntosh, D.P., Schnitzer, J.E., 1998. Dynamin at the neck of caveolae mediates their budding to form transport vesicles by GTP-driven fission from the plasma membrane of endothelium. J. Cell Biol. 141, 101–114.

Pasch, A., Kupper, J.H., Wolde, A., Kandolf, R., Selinka, H.C., 1999. Comparative analysis of virus-host cell interactions of haemagglutinating and non-haemagglutinating strains of coxsackievirus B3. J. Gen. Virol. 80, 3153–3158.

Pelkmans, L., Helenius, A., 2003. Insider information: what viruses tell us about endocytosis. Curr. Opin. Cell Biol. 15, 414–422.

Pho, M.T., Ashok, A., Atwood, W.J., 2000. JC virus enters human glial cells by clathrin-dependent receptor-mediated endocytosis. J. Virol. 74, 2288–2292.

Sabharanjak, S., Sharma, P., Parton, R.G., Mayor, S., 2002. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. Dev. Cell 2, 411–423.

Schmidtke, M., Selinka, H.C., Heim, A., Jahn, B., Tonew, M., Kandolf, R., Stelzner, A., Zell, R., 2000. Attachment of coxsackievirus B3 variants to various cell lines: mapping of phenotypic differences to capsid protein VP1. Virology 275, 77–88.

Selinka, H.C., Wolde, A., Pasch, A., Klingel, K., Schnorr, J.J., Kupper, J.H., Lindberg, A.M., Kandolf, R., 2002. Comparative analysis of two coxsackievirus B3 strains: putative influence of virus-receptor interactions on pathogenesis. J. Med. Virol. 67, 224–233.

Sever, S., Damke, H., Schmid, S.L., 2000. Dynamin:GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis. J. Cell Biol. 150, 1137–1148.

Shieh, J.T., Bergelson, J.M., 2002. Interaction with decay-accelerating factor facilitates coxsackievirus B infection of polarized epithelial cells. J. Virol. 76, 9474–9480.

Sieczkarski, S.B., Whittaker, G.R., 2002. Dissecting virus entry via endocytosis. J. Gen. Virol. 83, 1535–1545.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J.M., Brech, A., Callaghan, J., Toh, B.H., Murphy, C., Zerial, M., Stenmark, H., 1998. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature 394, 494–498.

Superti, F., Seganti, L., Ruggeri, F.M., Tinari, A., Donelli, G., Orsi, N., 1987. Entry pathway of vesicular stomatitis virus into different host cells. J. Gen. Virol. 68, 387–399.

Tomko, R.P., Xu, R., Philipson, L., 1997. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. U.S.A. 94, 3352–3356.

Triantafilou, K., Triantafilou, M., 2004. Lipid-raft-dependent coxsackievirus B4 internalization and rapid targeting to the Golgi. Virology 326, 6–19.

Wang, X., Bergelson, J.M., 1999. Coxsackievirus and adenovirus receptor cytoplasmic and transmembrane domains are not essential for coxsackievirus and adenovirus infection. J. Virol. 73, 2559–2562.

Zaragoza, C., Ocampo, C.J., Saura, M., McMillan, A., Lowenstein, C.J., 1997. Nitric oxide inhibition of coxsackievirus replication in vitro. J. Clin. Invest. 100, 1760–1767.